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14. ABSTRACT
The IGF axis is known to play an important role in the epidemiology of many tumors. IGFBP-3 promotes apoptosis in cancer cells by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. We demonstrate that phosphorylation of IGFBP-3 (S156) by DNA-PK enhances its nuclear accumulation, and is essential for its ability to interact with RXR and induce apoptosis in cultured prostate cancer cells. Indeed, IGFBP-3-S156A is completely unable to induce apoptosis in 22RV1 prostate cancer cells. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. We mapped two potential CK2 phosphorylation sites in IGFBP-3: S167 and S175. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells. WtIGFBP-3 and IGFBP-3-S175A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. Together, these data reveal two key regulatory phosphorylation sites in the central region of IGFBP-3. Phosphorylation of S156 by DNA-PK promotes apoptosis, whilst phosphorylation of S167 by CK2 limits the ability of IGFBP-3 to induce apoptosis.. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

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IGFBP-3, phosphorylation

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Introduction

The activity of IGF-I and -II is regulated by a family of six high affinity binding proteins. IGFBP-3 is the most abundant of the IGFBPs in serum, where it forms a ternary complex with acid labile subunit (ALS) and IGF (1). In addition to its role in regulating IGF action, IGFBP-3 exerts many IGF-independent effects to inhibit cell proliferation and enhance apoptosis in many cell types, including prostate (2) and breast (3-5) cancer. However, little is understood about the cellular mechanisms regulating IGFBP-3 action. IGFBP-3 is subject to post-translational modifications such as glycosylation and proteolysis, and also contains consensus phosphorylation sites for a variety of protein kinases. We investigated the significance of phosphorylation for the cellular actions of IGFBP-3 in prostate cancer. We identify phosphorylation to be a critical step in the growth-inhibitory and apoptosis-promoting actions of IGFBP-3.

Body

Statement of Work Task 1

DNA constructs have successfully been made in which the DNA-PK phosphorylation sites have been mutated to Alanine. An additional construct of IGFBP-3 with the signal peptide removed has been created.

Transfection of all constructs in to both LAPC4 and 22RV1 cells has been optimized and verified.

Statement of Work Task 2

The relevance of DNA-PK phosphorylation for IGFBP-3 action has been comprehensively analysed and published. For detailed descriptions of the role of DNA-PK phosphorylation for IGFBP-3 action, please refer to our manuscript recently published in Cancer Research (6). Briefly, we reveal that in the absence of active DNA-PK, IGFBP-3 has reduced nuclear localization and is unable to interact with its nuclear binding partner RXRα. It is also unable to exert its growth inhibitory or apoptosis-inducing actions in LAPC4 or 22RV1 prostate cancer cells. In addition, we identify S156 as the site specifically phosphorylated by DNA-PK. Please note that the methodology for experiments described below is as described in the above referenced manuscript (6).

In addition to the role of DNA-PK phosphorylation, the role of other protein kinases in regulating IGFBP-3 action are currently being assessed. When the protein sequence of the central region of IGFBP-3 is studied using bioinformatics (7), multiple putative phosphorylation sites are identified in the region surrounding Ser-156 (figure 1). These include sites for many kinases, including CK2. CK2 (formerly casein kinase 2) is a highly conserved and ubiquitously expressed kinase which plays a key role in the regulation of cell growth, proliferation and apoptosis. Importantly, CK2 is dysregulated in most cancers, including prostate (for review, see 8). To investigate the relevance of CK2 phosphorylation in the regulation of IGFBP-3-induced apoptosis, we utilized two chemical inhibitors against CK2, TBB and DMAT. We demonstrate that incubation of IGFBP-3 with low doses of either inhibitor (900 nM and 100 nM, respectively), insufficient to induce apoptosis, was adequate to reduce the serine phosphorylation of IGFBP-3, as demonstrated by immunoprecipitation of IGFBP-3 followed by phospho-serine-specific immunoblotting (figure 2A). Surprisingly, when CK2 activity is inhibited by either chemical inhibitor, exogenously added IGFBP-3 had significantly enhanced ability to induce apoptosis in

both 22RV1 and LAPC4 prostate cancer cells, as determined by caspase-3/-7 activity (figure 2B). These data were confirmed using siRNA against CK2α. Transfection of LAPC4 cells with a verified CK2α duplex of siRNA (STEALTH, Invitrogen) resulted in CK2α protein levels which were reduced by approximately 75%, as determined by immunoblotting (figure 3A). IGFBP-3 exogenously added to cells which had been transfected with CK2α siRNA had an ability to induce apoptosis which was significantly greater compared with IGFBP-3 which had been added to cells transfected with scrambled siRNA control (figure 3B). Since phosphorylation by DNA-PK was previously identified to regulate the intracellular localization of IGFBP-3, we investigated whether the enhanced apoptosis induction observed with reduced CK2 activity correlated with enhanced nuclear localization of IGFBP-3. LAPC4 cells were incubated with 900 nM TBB for 24 hours, and cytoplasmic and nuclear fractions were isolated. Consistent with the nuclear localization of IGFBP-3 being critical for apoptosis induction in our cell systems, enhanced nuclear localization of IGFBP-3 was observed when CK2 activity was inhibited (figure 4).

In order to identify the specific site in IGFBP-3 responsible for the enhanced apoptosis induction observed in the absence of active CK2, the two putative CK2 phosphorylation sites in the central region of IGFBP-3 (S167, S175) were mutated to alanine by site directed mutagenesis. The resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells, and their ability to induce apoptosis was compared with wild type IGFBP-3. Transfection with wtIGFBP-3 or IGFBP-3/S175A caused the induction of apoptosis which was significantly enhanced by incubation with a CK2 inhibitor (figure 5). In contrast, transfection with IGFBP-3/S167A caused an enhanced apoptosis induction which was unaffected by the presence of a CK2 inhibitor, suggesting that S167 of IGFBP-3 may be directly phosphorylated by CK2, and that this phosphorylation event limits the ability of IGFBP-3 to induce apoptosis. This leads to a model whereby two distinct phosphorylation events in the central region of IGFBP-3 regulate its ability to induce apoptosis. Phosphorylation of S156 by DNA-PK is a pro-apoptotic event, and is essential for IGFBP-3-induced apoptosis in 22RV1 and LAPC4 prostate cancer cells. Conversely, phosphorylation of S167 by CK2 acts as an anti-apoptotic event, and limits (but not completely inhibits) the ability of IGFBP-3 to induce apoptosis.

To compare the ability of wtIGFBP-3 and IGFBP-3/S167A to influence cell growth, LAPC4 cells were transfected with control vector, wtIGFBP-3 and IGFBP-3/S167A. When cell proliferation

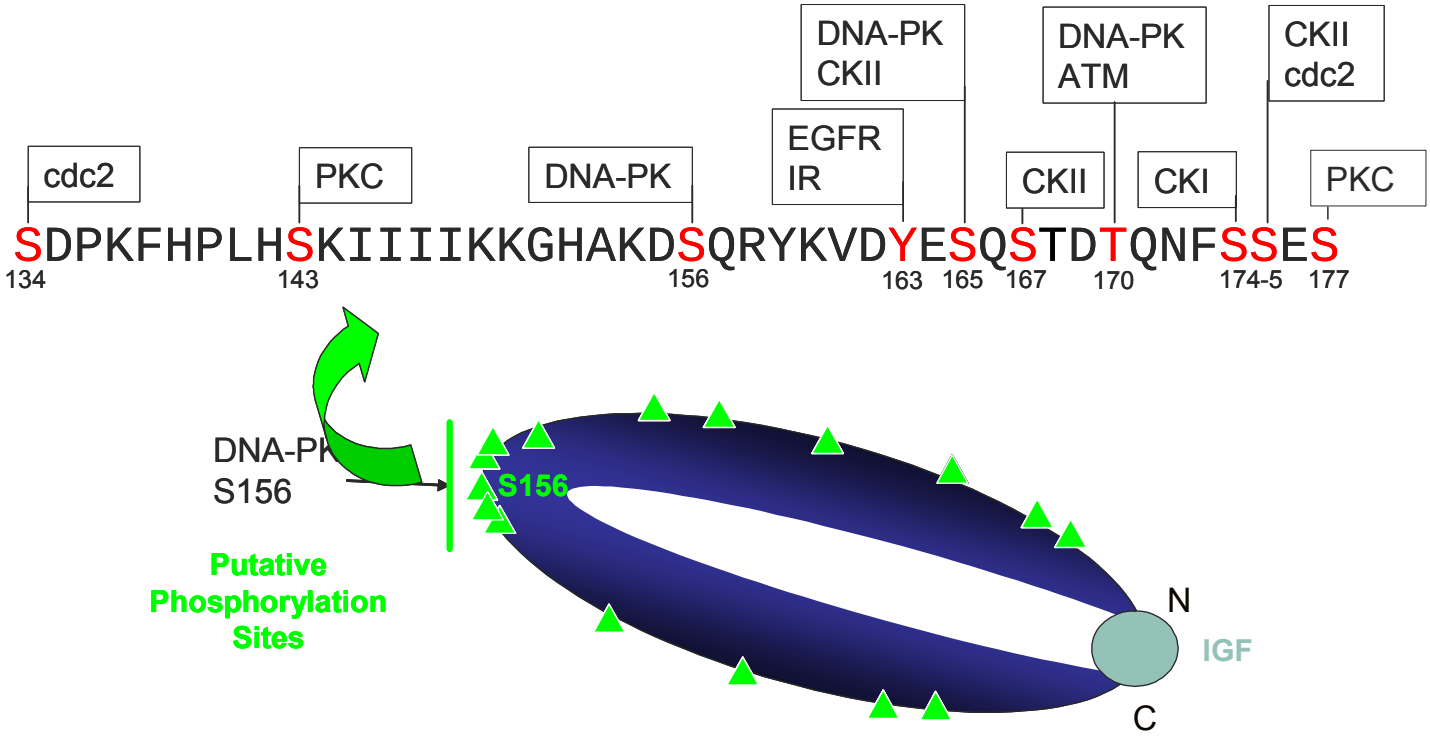
was assessed by both MTS assay and BrdU incorporation, IGFBP-3 caused a 15-20% reduction in the growth of LAPC4 cells, consistent with previous findings (Figure 6). However IGFBP-3/S167A caused no further reduction in cell number/growth, suggestion that phosphorylation by CK2 is important only for the regulating the apoptotic action of IGFBP-3.

To determine whether the pro- or anti-apoptotic phosphorylation event is dominant, we utilized a combination of chemical inhibitors (NU7026 for DNA-PK and TBB for CK2) and our non-phosphorylateable mutants (S156 for DNA-PK and S167 for CK2). As described, the transfection of LAPC4 or 22RV1 cells with wtIGFBP-3 causes the induction of apoptosis which is inhibited by NU7026 and enhanced by TBB. IGFBP-3/S165A is unable to induce apoptosis, and is completely unaffected by the presence of NU7026. However, in the presence of the CK2 inhibitor, IGFBP-3/S156A is able to induce apoptosis to the same extent as wtIGFBP-3, suggesting that preventing the activity of CK2 allows IGFBP-3 to overcome the inhibition caused by the absence of DNA-PK phosphorylation (figure 7). However, incubation with NU7026 was unable to inhibit the apoptosis induced by IGFBP-3/S167A, suggesting that the oncogenic phosphorylation event of CK2 is dominant over the tumor-suppressive action of DNA-PK. Together, these data provide a model whereby the apoptotic actions of IGFBP-3 are regulated by distinct pro- and anti-apoptotic phosphorylation events. The pro-apoptotic phosphorylation of S156 by DNA-PK is necessary for IGFBP-3-induced apoptosis in LAPC4 and 22RV1 prostate cancer cells. In addition, the anti-apoptotic phosphorylation event by CK2 limits the ability of IGFBP-3 to induce apoptosis (figure 8).

Statement of work Task 3

The work defined by specific aim 3 of the research proposal was to create prostate specific IGFBP-3 transgenic mice, expressing wtIGFBP-3 and IGFBP-3/S156A, and to cross the resulting mice in to the TRAMP mouse model of prostate cancer. However, the laboratory is currently in the process of transferring from the TRAMP model (in which tumors develop very quickly) to the Myc model of prostate cancer, in which tumors have a slower development, enabling easier investigation of the earlier stages of prostate cancer. In addition, long running experiments in the laboratory have limited the available space within the mouse facility. These studies have consequently not yet begun.

Figure 1



Cartoon demonstrating the phosphorylation sites identified in the region surrounding S156 of IGFBP-3. Putative phospho-acceptor residues are colored red, and potential kinases are demonstrated in boxes.

Figure 2

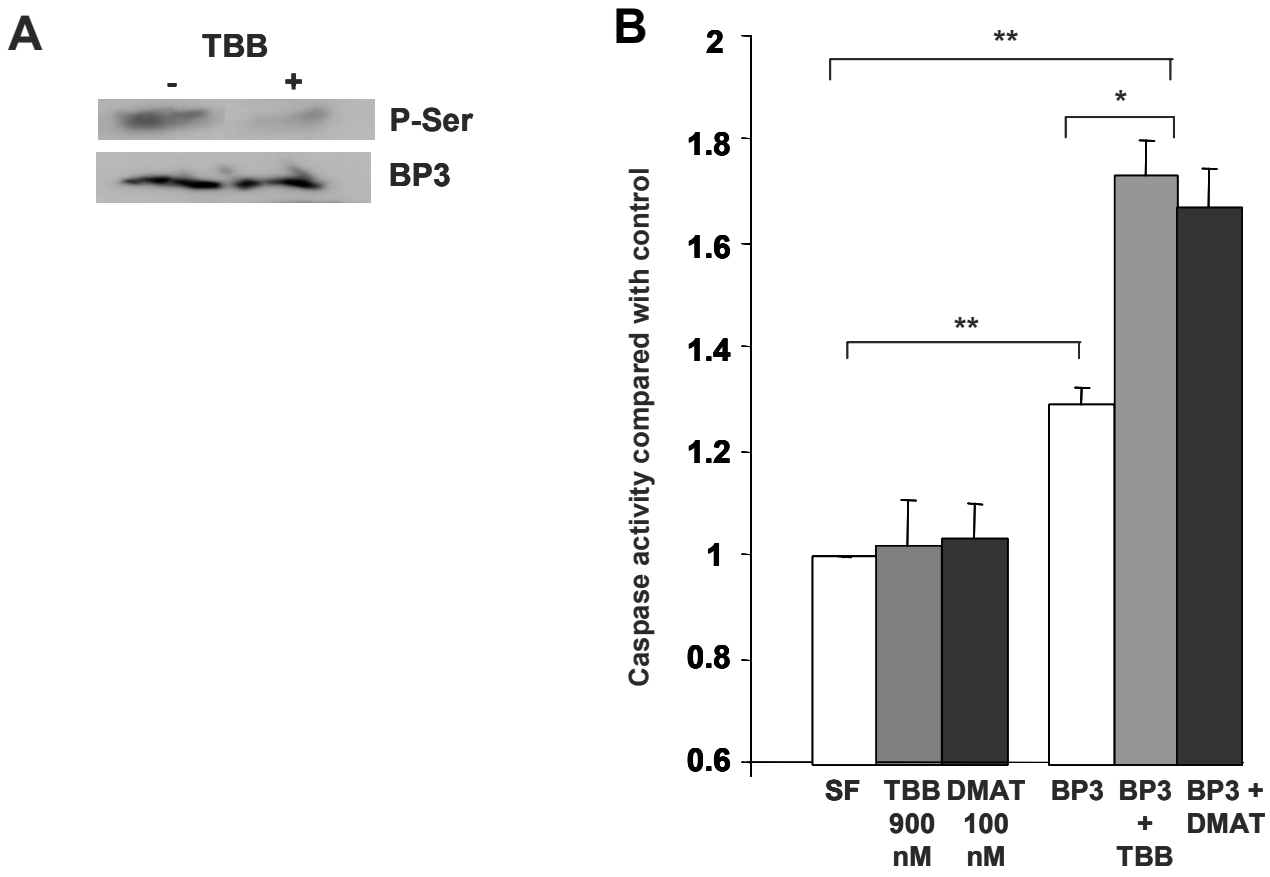


Figure 2 - Incubation of IGFBP-3 with CK2 inhibitors results in decreased serine phosphorylation and increased ability to induce apoptosis in LAPC4 prostate cancer cells. LAPC4 cells were incubated with 900 nM TBB or 100 nM DMAT in serum free media for 24 h. *A*, immunoprecipitation of IGFBP-3 followed by immunoblotting for P-Ser and IGFBP-3. *B,* caspase-3/-7 activity after additional incubation for 24 h with 1 ug/ml recombinant IGFBP-3. Significance that mean is different from 1: * $P<0.05$; ** $P<0.01$.

Figure 3

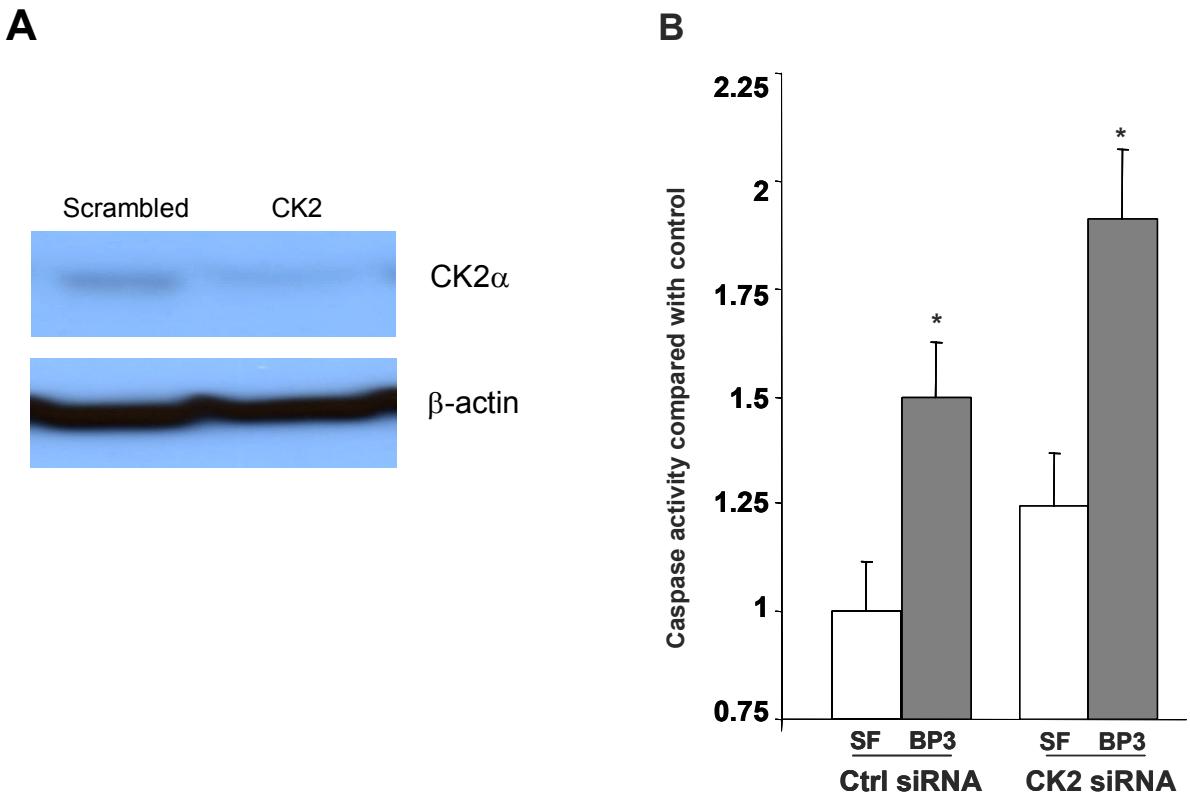


Figure 3 – Treatment of LAPC4 cells with CK2α siRNA enhances the ability of IGFBP-3 to induce apoptosis. LAPC4 cells were transfected with CK2α or scrambled siRNA for 72 h. *A*, immunoblot for CK2α or β-actin (loading control) to demonstrate reduced protein expression of CK2α. *B*, Caspase-3/-7 assay determined by cleavage of a luminescent substrate after incubation for 24 h in SF media with 1 μg/ml IGFBP-3. Significance that mean is different from 1: * $P<0.05$

Figure 4

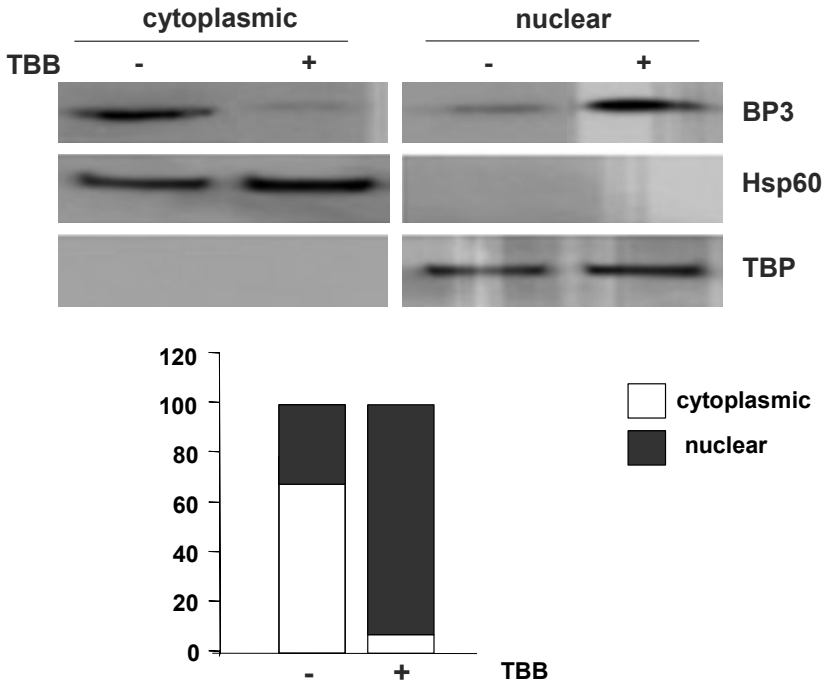


Figure 4 – Enhanced nuclear localization of IGFBP-3 in the absence of active CK2α. 22RV1 cells were incubated with 900 nM TBB for 24 h in serum free media. The localization of endogenous IGFBP-3 was assessed by cellular fractionation followed by immunoblotting for IGFBP-3, Hsp60 (cytoplasmic fraction control) and TBP (tata binding protein, nuclear fraction control). Lower panel demonstrates cytoplasmic/nuclear localization of IGFBP-3 expressed as percentage of total IGFBP-3.

Figure 5

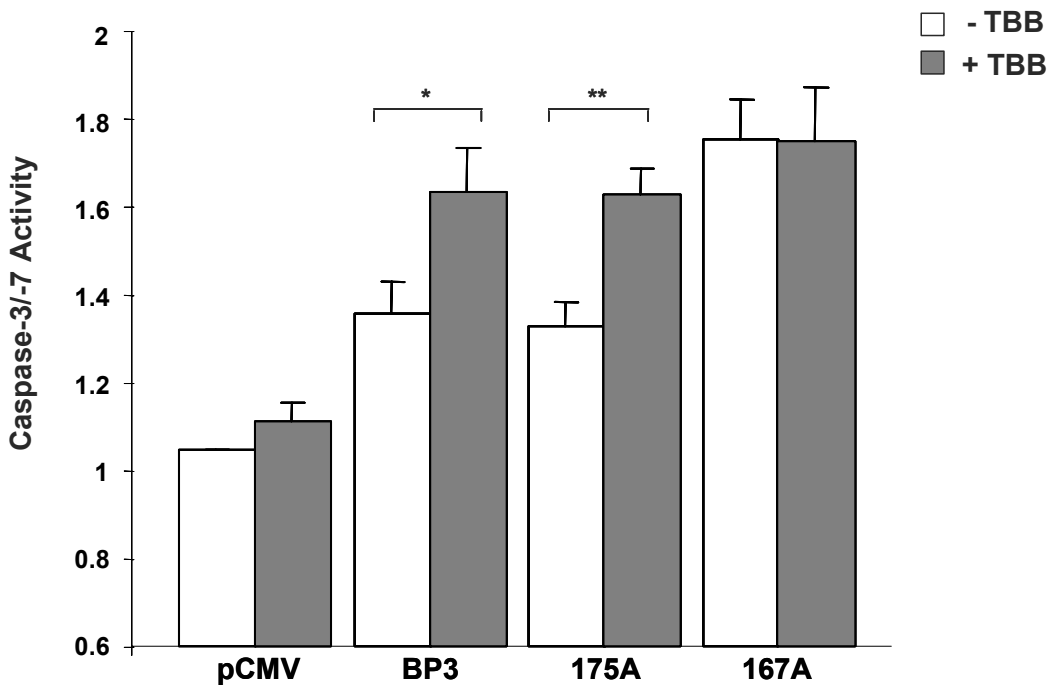


Figure 5 – IGFBP-3/S167A has enhanced ability to induce apoptosis and is unaffected by incubation with TBB. LAPC4 cells were transiently transfected with control vector (pCMV), wtIGFBP-3 (BP3), IGFBP-3/S175A (175A) or IGFBP-3/S167A (167A). 48 h after transfection, cells were transferred to serum free media in the presence or absence of 900 nM TBB. Apoptosis induction was assessed by incubation with a luminometric caspase-3/-7 substrate. * $P<0.05$; ** $P<0.01$

Figure 6

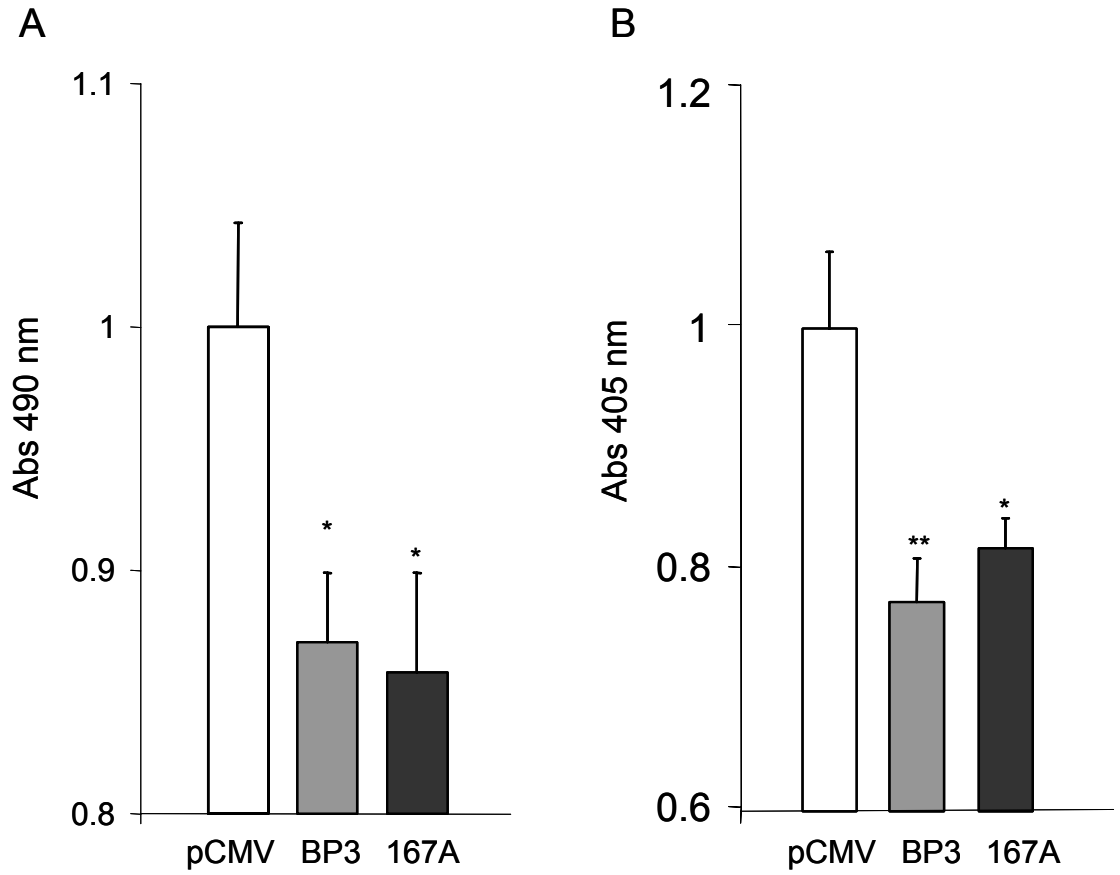


Figure 6 – Phosphorylation of S167A does not affect growth inhibition by IGFBP-3 LAPC4 cells were transiently transfected with control vector (pCMV), wtIGFBP-3 (BP3), or IGFBP-3/S167A (167A). *A*, 48 h after transfection, cells were transferred to serum free media for 72 h. Cell growth was assessed by MTT assay. Significance that mean is different from 1 * $P<0.05$. *B*, cells were incubated in serum free media for 24 hours, and BrdU incorporation was assessed using ELISA assay. Significance that mean is different from 1 * $P<0.05$; ** $P<0.01$.

Figure 7

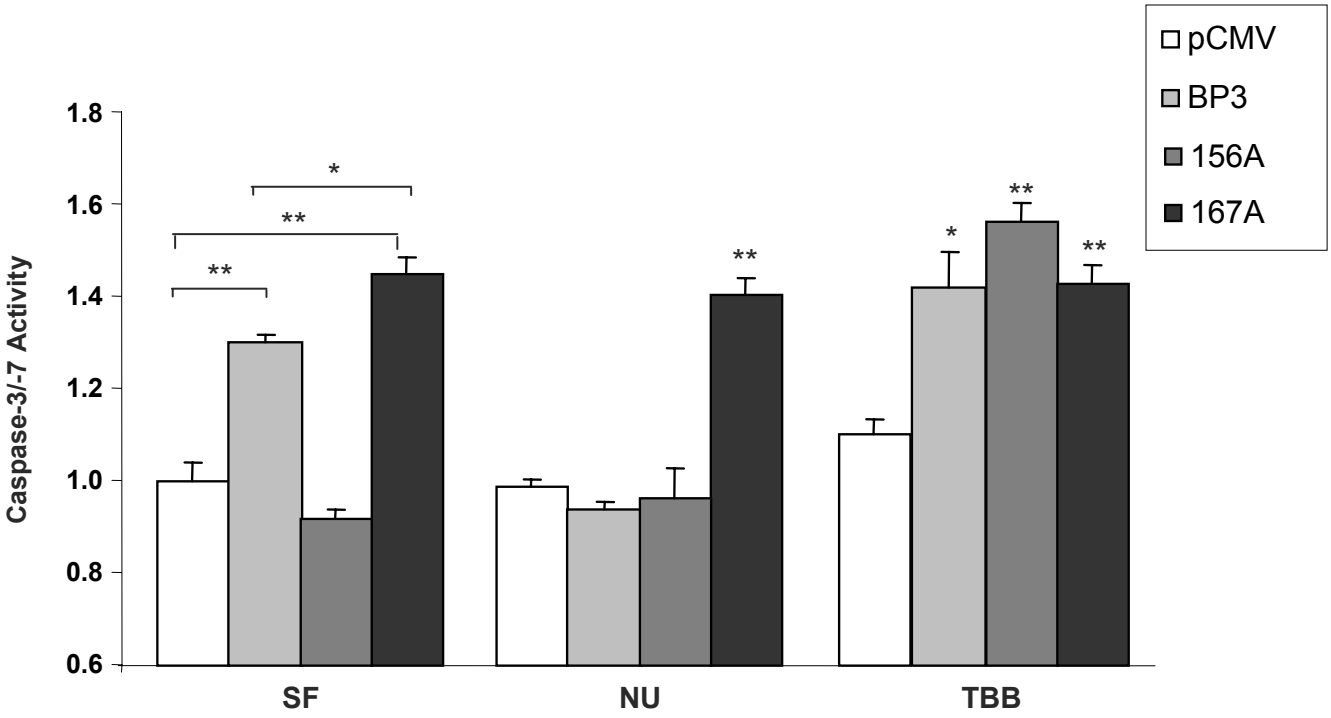
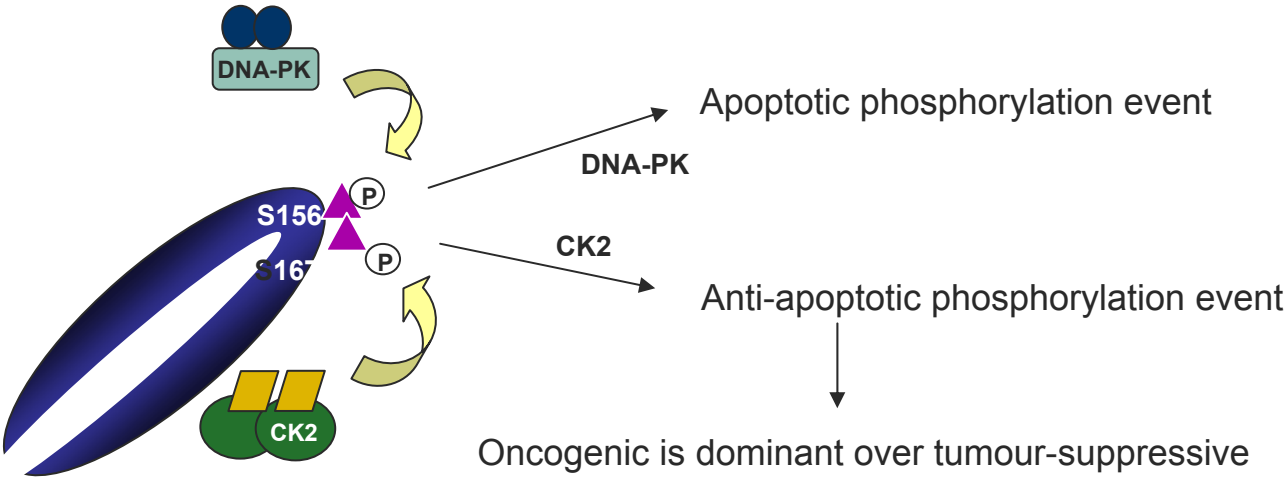


Figure 7 - Phosphorylation by CK2 is dominant over DNA-PK. LAPC4 cells were transfected with control vector (pCMV), wtIGFBP-3 (BP3), IGFBP-3/S156A (156A) or IGFBP-3/S167A (167A). 72 h after transfection, cells were transferred to serum free media in the presence or absence of 100 μM NU7026 or 900 nM TBB. Apoptosis induction was assessed after 24 h by cleavage of luminometric caspase-3/-7 substrate. * $P<0.05$; ** $P<0.01$.

Figure 8



Cartoon demonstrating the regulation of IGFBP-3 induced apoptosis by DNA-PK and CK2. Phosphorylation of S156 by DNA-PK acts as a pro-apoptotic event, allowing IGFBP-3 to induce apoptosis. Conversely, phosphorylation of S167 by CK2 is anti-apoptotic, limiting the ability of IGFBP-3 to induce apoptosis. The oncogenic phosphorylation event is dominant over the tumor-suppressive action.

Key Research Accomplishments

- Identification of phosphorylation by DNA-PK to be essential for IGFBP-3 mediated apoptosis
- Identification of Ser-156 as a critical residue for IGFBP-3-induced apoptosis
- Production of IGFBP-3/S156A, an expression vector of IGFBP-3 which is unable to induce apoptosis
- Identification of CK2 as an IGFBP-3 kinase, and discovery of S167 as the residue specifically phosphorylated
- Identification of IGFBP-3/S167A as an improved-potency apoptosis inducing protein, and production of the expression plasmid
- Recognition of phosphorylation as the dual-specificity mechanism for the regulation of IGFBP-3-induced apoptosis

Reportable Outcomes

- Phosphorylation by DNA-Dependent Protein Kinase Is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3. Laura J. Cobb, Bingrong Liu, Kuk-Wha Lee, and Pinchas Cohen. Cancer Res 2006 66: 10878-10884.

- Abstract, ENDO 2006 (poster presentation). Phosphorylation of Ser-156 by DNA-PK is functionally critical for apoptosis induction by IGFBP-3. Laura J Cobb, Bingrong Liu, Kuk-Wha Lee and Pinchas Cohen.

- Abstract, 3rd GRS/IGF joint symposium (selected for oral presentation). Site specific phosphorylation by intracellular kinases determines the apoptotic activity of IGFBP-3. Laura J Cobb, Bingrong Liu, Kuk-Wha Lee and Pinchas Cohen

- Abstract submitted for AACR 2007. Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3 in Prostate Cancer. Laura J Cobb, Satomi Koyama and Pinchas Cohen

- Abstract submitted for GRS "IGFs in physiology and disease" 2007. Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3. Laura J Cobb, Satomi Koyama and Pinchas Cohen

- IGFBP-3/S156A, an expression plasmid developed of a form of IGFBP-3 unable to induce apoptosis

- IGFBP-3/S167A, an expression plasmid developed of IGFBP-3 with increased potential to induce apoptosis

Conclusions

The IGF axis is known to play an important role in the epidemiology of many tumors, including prostate, lung and breast cancers. IGFBP-3 promotes apoptosis in cancer cells by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. Proteomic and bioinformatic analysis of IGFBP-3 reveals multiple consensus phosphorylation sites for kinases including CK2, PKA, PKC and cdc2. We have previously reported that phosphorylation of IGFBP-3 (S156) by DNA-PK enhances its nuclear accumulation, and is essential for its ability to interact with RXR and induce apoptosis in cultured prostate cancer cells. Indeed, IGFBP-3-S156A is completely unable to induce apoptosis in 22RV1 prostate cancer cells. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. Using web-based proteomics software, we mapped three potential CK2 phosphorylation sites in IGFBP-3: S167, S175 and S177. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells. WtIGFBP-3, IGFBP-3-S175A and IGFBP-3-S177A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. Interestingly, IGFBP-3-S167A was able to induce apoptosis even in the absence of active DNA-PK, and IGFBP-3-S156A was able to induce apoptosis when CK2 activity was inhibited chemically or by using siRNA. Together, these data reveal two key regulatory phosphorylation sites in the central region of IGFBP-3. Phosphorylation of S156 by DNA-PK promotes apoptosis, whilst phosphorylation of S167 by CK2 limits the ability of IGFBP-3 to induce apoptosis in prostate cancer. Interestingly, our data suggest that the anti-apoptotic phosphorylation event induced by CK2 is dominant. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

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(Mostly additional references not referenced in appended manuscript)

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6. Phosphorylation by DNA-Dependent Protein Kinase Is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3. Cobb LJ, Liu B, Lee KW, and Cohen P. Cancer Res 2006;66:10878-10884.
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1. **Appendices**

    1. Updated biosketch

    2. Abstract. ENDO 2006

    3. Abstract – GRS/IGF 3rd joint meeting 2006

    4. Abstract – Gordon Research Conference (IGFs in physiology and disease) 2007

    5. Abstract – AACR 2007

    6. Manuscript. Phosphorylation by DNA-Dependent Protein Kinase Is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3. Laura J. Cobb, Bingrong Liu, Kuk-Wha Lee, and Pinchas Cohen. Cancer Res 2006 66: 10878-10884.

Appendix 1 – Updated Biosketch

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Laura Cobb	Post-Doctoral Researcher

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Newcastle-upon-Tyne, UK	BSc	1998-2001	Biochemistry
The Babraham Institute/University of Cambridge, Cambridge, UK	PhD	2001-2004	IGFBP-5 and intracellular signaling during myogenesis
University of California, Los Angeles, USA		2004-	IGFBP-3 in prostate cancer

Professional Experience

2000 **Research Assistant**, Department of Biochemistry and Genetics, University of Newcastle upon Tyne, Newcastle-upon-Tyne, UK

2001-2004 **Graduate Student**, Signalling Programme, The Babraham Institute/Cambridge University, Cambridge, UK

2004- **Post-Doctoral Researcher**, Department of Pediatrics, University of California, Los Angeles

Honors and Awards

2001 First prize in Biochemistry examination finals

2003 Travel prize at Gordon Research Conference

2005 US Department of Defense Prostate Cancer Post-Doctoral Fellowship Award

2006 Travel award at GRS/IGF meeting, Kobe Japan

Presentations Given

2003 Gordon Research Conference, Ventura, CA. The IGF-dependent and -independent roles of IGFBP-5 in myogenesis (poster and oral).

2003 The Babraham Institute Annual Lab Talks. IGFBP-5 is an IGF-independent survival factor in myogenesis (poster).

2004 The Babraham Institute Annual Lab Talks. IGF-independent roles of IGFBP-5 during myogenesis (oral).

2004 Signalling Programme Seminar Series, The Babraham Institute, UK. IGFBP-5: an Apoptotic Switch During Myogenesis (oral)

2004 UK Endocrine Society Annual Meeting. IGF-independent acceleration of myogenesis by IGFBP-5: modulation of Akt, Bcl-x_L and Bad signalling pathways (oral).

2005 ENDO 2005. Phosphorylation of IGFBP-3 by DNA-PK is required for its apoptotic effects (poster)

2006 ENDO 2006. Phosphorylation of Ser-156 by DNA-PK is functionally critical for apoptosis induction by IGFBP-3 (poster)

2006 GRS/IGF 3rd joint meeting. Site specific phosphorylation by intracellular kinases determines the apoptotic activity of IGFBP-3. (oral)

Publications

Cobb LJ, Salih DAM, Gonzalez I, Tripathi G, Carter EJ, Lovett F, Holding C and Pell JM (2004). Partitioning of IGFBP-5 actions during myogenesis: IGF-independent ani-apoptotic function. *J. Cell Sci.* 117 1737-1746

Gonzalez, I, Tripathi, G, Carter, EJ, **Cobb, LJ**, Salih, DAM, Lovett, FA, Holding, C and Pell, JM (2004). Akt2, a Novel Functional Link between p38 Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase Pathways in Myogenesis. *Mol Cell Biol* **24** 3607-22.

Salih, DAM, Tripathi, G, Holding, C, Szestak, TAM, Gonzalez, MI, Carter, EJ, **Cobb, LJ**, Eisemann, JE and Pell, JM (2004). *Insulin-like growth factor-binding protein 5 (Igfbp5)* compromises survival, growth, muscle development, and fertility in mice. *Proc Natl Acad Sci U S A* **101** 4314-9.

Pell, JM, Salih, DA, **Cobb, LJ**, Tripathi, G, Drozd, A (2005). The role of insulin-like growth factor binding proteins in development. *Rev Endocr Metab Disord* 6(3) 189-98.

Cobb LJ, Liu B, Lee KW, Cohen P (2006). Phosphorylation by DNA-Dependent Protein Kinase is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3. *Cancer Res*. 66: 10878-10884.

Lovett FA, Gonzalez I, Salih DA, **Cobb LJ**, Tripathi G, Cosgrove RA, Murrell A, Kilshaw P, Pell JM (2006). Convergence of Igf2 expression and adhesion signalling via RhoA and p38 MAPK enhances myogenic differentiation. *J Cell Sci* in press (Epub ahead of print)

Manuscript under review

Lee KW, **Cobb LJ**, Ma L, Liu B, Milbrandt J, Cohen P (2006). Contribution of the orphan nuclear receptor Nur77 to the apoptotic action of IGFBP-3. Submitted to *Carcinogenesis*

Manuscripts under Preparation

Cobb LJ, Drozd A, Gonzalez I, Tripathi G, Lovett FA, Salih DAM, and Pell JM. IGF-independent acceleration of myogenesis by IGFBP-5: modulation of Akt, Bcl-x_L and Bad signalling pathways.

Cobb LJ, Drozd A, Gonzalez I, Tripathi G, Lovett FA, Salih DAM, and Pell JM. IGFBP-5: an apoptotic switch during myogenesis.

Tripathi G, Salih, SAM, **Cobb LJ**, Gonzalez I, Lovett FA and Pell, JM. Overexpression of non-IGF binding IGFBP-5 *in vivo* demonstrates its major IGF-independent role in growth and development.

Appendix 2 – Abstract for ENDO 2006

Phosphorylation of Ser-156 by DNA-PK is functionally critical for apoptosis induction by IGFBP-3

Laura J Cobb, Bingrong Liu, Kuk-Wha Lee, and Pinchas Cohen
UCLA

IGFBP-3 promotes apoptosis by both IGF-dependent and -independent mechanisms. We have previously shown in prostate cancer (CaP) cells that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are essential for apoptosis induction. Protein sequence analysis of IGFBP-3 reveals multiple putative phosphorylation sites, and *in vitro* phosphorylation by DNA-dependent protein kinase (DNA-PK) has been reported. However, the functional relevance of phosphorylation for the physiological actions of IGFBP-3 is unknown. We set out to define the significance of phosphorylation of IGFBP-3 by DNA-PK for its growth inhibitory and pro-apoptotic actions *in vitro* using 2 systems. First, we employed a specific ATP-competitive inhibitor for DNA-PK (NU7026) in cultured CaP cells; and second, we studied a paired cell system of glioblastoma cell lines that either lack (M059J) or express DNA-PK (M059K). We demonstrate that, in the presence of normal DNA-PK activity, IGFBP-3 promotes apoptosis and is growth inhibitory. However, these functions are completely abrogated in the absence of DNA-PK, as assessed by caspase-3/-7 activity and MTT assay, suggesting that phosphorylation of IGFBP-3 may be critical for specific cellular actions. In the absence of DNA-PK activity, IGFBP-3 has reduced nuclear accumulation, and is no longer able to bind to its nuclear binding partner RXRα, suggesting a partial mechanism for the loss of apoptosis induction observed. We assessed the importance of the three potential DNA-PK phosphorylation sites, S156, S165 and T170A, in IGFBP-3 using PCR based site-directed mutagenesis. When transfected in to 22RV1 CaP cells, the non-phosphor-able IGFBP-3-S156A mutant was no longer able to promote apoptosis, assessed by DNA fragmentation ELISA and caspase-3 activity, and exhibited reduced nuclear accumulation, assessed by cell fractionation and IGFBP-3 immunoblotting. In contrast, the IGFBP-3-S165A and IGFBP-3-T170A mutants induced apoptosis to the same extent as wild-type IGFBP-3, and showed normal nuclear accumulation. These studies reveal a novel regulatory mechanism for the cellular actions of IGFBP-3 in prostate cancer, and demonstrate phosphorylation of S156 to be functionally critical in its apoptosis-inducing actions.

Appendix 3 – Abstract for GRS/IGF 2006

Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3

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IGFBP-3 promotes apoptosis by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. Proteomic and bioinformatic analysis of IGFBP-3 reveals multiple consensus phosphorylation sites for kinases including CK2, PKA, PKC and cdc2. In addition, *in vitro* phosphorylation by DNA-dependent protein kinase (DNA-PK) has been reported. However, the significance of phosphorylation in regulating IGFBP-3 action is unknown. Using a specific ATP-competitive inhibitor for DNA-PK (NU7026) in cultured CaP cells, and a paired system of glioblastoma cell lines that either lack (M059J) or express DNA-PK (M059K), we demonstrate that phosphorylation by DNA-PK is essential for the growth-inhibitory and apoptosis-inducing actions of IGFBP-3. Specifically, preventing such phosphorylation caused reduced nuclear accumulation of IGFBP-3, and complete loss of interaction with RXRα. To assess the importance of the potential DNA-PK phosphorylation sites, S156, S165 and T170, we mutated each site to Ala to prevent their phosphorylation. When transfected in to 22RV1 and LAPC4 cells, wtBP3, BP3-S165A and BP3-T170A showed predominantly nuclear localization, and induced apoptosis that could be inhibited by NU7026. In contrast, IGFBP-3-S156A was unable to promote apoptosis and exhibited reduced nuclear accumulation, suggesting that its phosphorylation is a crucial step in IGFBP-3 action. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Whilst inhibiting PKA, PKC and cdc2 had no effect on IGFBP-3-induced apoptosis, preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. Using web-based proteomics software, we mapped three potential CK2 phosphorylation sites in IGFBP-3: S167, S175 and S177. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 cells. WtIGFBP-3, IGFBP-3-S175A and IGFBP-3-S177A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

Appendix 4 – Abstract for GRC IGF Meeting 2007

Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3

Laura Cobb, Satomi Koyama, Pinchas Cohen

IGFBP-3 promotes apoptosis by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. Proteomic and bioinformatic analysis of IGFBP-3 reveals multiple consensus phosphorylation sites for kinases including CK2, PKA, PKC and cdc2. We have previously reported that phosphorylation of IGFBP-3 (S156) by DNA-PK enhances its nuclear accumulation, and is essential for its ability to interact with RXR and induce apoptosis in cultured prostate cancer cells. Indeed, IGFBP-3-S156A is completely unable to induce apoptosis in 22RV1 prostate cancer cells. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Whilst inhibiting PKA, PKC and cdc2 had no effect on IGFBP-3-induced apoptosis, preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. Using web-based proteomics software, we mapped three potential CK2 phosphorylation sites in IGFBP-3: S167, S175 and S177. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 cells. WtIGFBP-3, IGFBP-3-S175A and IGFBP-3-S177A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. Interestingly, IGFBP-3-S167A was able to induce apoptosis even in the absence of active DNA-PK, and IGFBP-3-S156A was able to induce apoptosis when CK2 activity was inhibited chemically or by using siRNA. Together, these data reveal two key regulatory phosphorylation sites in the central region of IGFBP-3. Phosphorylation of S156 by DNA-PK promotes apoptosis, whilst phosphorylation of S167 by CK2 limits the ability of IGFBP-3 to induce apoptosis. Interestingly, our data suggest that the anti-apoptotic phosphorylation event induced by CK2 is dominant. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

Appendix 5 – Abstract for AACR 2007

Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3 in Prostate Cancer

Laura Cobb, Satomi Koyama, Pinchas Cohen

The IGF axis is known to play an important role in the epidemiology of many tumors, including prostate, lung and breast cancers. IGFBP-3 promotes apoptosis in cancer cells by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. Proteomic and bioinformatic analysis of IGFBP-3 reveals multiple consensus phosphorylation sites for kinases including CK2, PKA, PKC and cdc2. We have previously reported that phosphorylation of IGFBP-3 (S156) by DNA-PK enhances its nuclear accumulation, and is essential for its ability to interact with RXR and induce apoptosis in cultured prostate cancer cells. Indeed, IGFBP-3-S156A is completely unable to induce apoptosis in 22RV1 prostate cancer cells. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. Using web-based proteomics software, we mapped three potential CK2 phosphorylation sites in IGFBP-3: S167, S175 and S177. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells. WtIGFBP-3, IGFBP-3-S175A and IGFBP-3-S177A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. Interestingly, IGFBP-3-S167A was able to induce apoptosis even in the absence of active DNA-PK, and IGFBP-3-S156A was able to induce apoptosis when CK2 activity was inhibited chemically or by using siRNA. Together, these data reveal two key regulatory phosphorylation sites in the central region of IGFBP-3. Phosphorylation of S156 by DNA-PK promotes apoptosis, whilst phosphorylation of S167 by CK2 limits the ability of IGFBP-3 to induce apoptosis in prostate cancer. Interestingly, our data suggest that the anti-apoptotic phosphorylation event induced by CK2 is dominant. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

Phosphorylation by DNA-Dependent Protein Kinase Is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3

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Abstract

Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) promotes apoptosis of cancer cells by both IGF-dependent and IGF-independent mechanisms. *In vitro* phosphorylation of IGFBP-3 by DNA-dependent protein kinase (DNA-PK) has been reported but with unknown functional relevance. Using a chemical inhibitor for DNA-PK in prostate cancer cells and a paired system of glioblastoma cell lines that either lack or express DNA-PK, we show that the apoptosis-promoting and growth-inhibitory actions of IGFBP-3 are completely abrogated in the absence of catalytically active DNA-PK. In the absence of DNA-PK activity, IGFBP-3 has reduced nuclear accumulation and is unable to bind its nuclear binding partner retinoid X receptor (RXR) α. We assessed the importance of the three potential DNA-PK phosphorylation sites in IGFBP-3 using PCR-based site-directed mutagenesis. When transfected into 22RV1 cells, IGFBP-3-S165A and IGFBP-3-T170A functioned in an identical manner to wild-type IGFBP-3 to induce apoptosis. In contrast, IGFBP-3-S156A was unable to promote apoptosis and exhibited reduced nuclear accumulation, suggesting a key role for DNA-PK-dependent phosphorylation in the regulation of IGFBP-3 action. These studies reveal a novel regulatory mechanism for the actions of IGFBP-3 in prostate cancer and show phosphorylation of Ser^{156} to be functionally critical in its apoptosis-inducing actions. (Cancer Res 2006; 66(22): 10878-84)

Introduction

The activity of insulin-like growth factor (IGF)-I and IGF-II is regulated by a family of six high-affinity binding proteins. IGF binding protein (IGFBP)-3 is the most abundant of the IGFBPs in serum, where it forms a ternary complex with acid labile subunit and IGF (1). In addition to its role in regulating IGF action, IGFBP-3 exerts many IGF-independent effects to inhibit cell proliferation and enhance apoptosis in many cell types, including prostate (2) and breast (3–5) cancers.

IGFBP-3 has been reported in the nucleus of many cell types and contains a nuclear localization sequence (NLS) that facilitates nuclear uptake (6–8). Extracellular IGFBP-3 is rapidly internalized via transferrin receptor and caveolin and is transported into the nucleus by importin-β (9, 10). Once localized to the nucleus, IGFBP-3 interacts with the nuclear receptor retinoid X receptor (RXR) α to promote apoptosis by a mechanism that involves the nucleo-mitochondrial shuttling of RXRα/Nur77 (11, 12). However, IGFBP-3 may function in different ways to induce apoptosis because IGFBP-3 lacking a functional NLS is reported to promote apoptosis in breast cancer cells (13). However, little is understood about the cellular mechanisms regulating IGFBP-3 action.

IGFBP-3 is subject to post-translational modifications, such as glycosylation and proteolysis, and also contains consensus phosphorylation sites for a variety of protein kinases. In particular, Ser^{111} and Ser^{113} have been described as phosphoacceptor residues possibly for CK2 (14, 15). Phosphorylation of these sites may affect the ability of IGFBP-3 to become glycosylated because the S111A/S113A double mutant showed a strongly reduced glycosylation pattern (14). Phosphorylation of IGFBP-3 at the cell membrane of T-47D cells was reported to enhance IGF binding (16). IGFBP-3 can also be phosphorylated by DNA-dependent protein kinase (DNA-PK) and cyclic AMP–dependent protein kinase A (PKA) after incubation with recombinant enzyme and [γ-^{32}P]ATP (17). DNA-PK is a predominantly nuclear serine/threonine protein kinase, which is activated in response to DNA damage. It plays a role in numerous cellular processes, including DNA double-strand break repair, V(D)J recombination, telomere maintenance, and gene transcription (18). DNA-PK phosphorylates many transcription factors *in vitro*, including p53, a tumor suppressor that also functions to regulate the transcription of IGFBP-3 (19). Exogenously added IGFBP-3 that had been phosphorylated by DNA-PK displayed enhanced nuclear accumulation in Chinese hamster ovary (CHO) cells and decreased IGF binding compared with the nonphosphorylated form (17).

We investigated the significance of phosphorylation by DNA-PK for the cellular actions of IGFBP-3 in prostate cancer. We identify phosphorylation to be a critical step in the growth-inhibitory and apoptosis-promoting actions of IGFBP-3. DNA-PK-mediated phosphorylation enhances the nuclear accumulation of IGFBP-3 and is critical for interactions with its nuclear binding partner RXRα. Moreover, we reveal that Ser^{156} is the phosphoacceptor residue for DNA-PK and that this phosphorylation event is crucial for IGFBP-3 to exert these effects.

Materials and Methods

Reagents. Recombinant nonglycosylated IGFBP-3 was provided by Insmed (Glen Allen, VA). Goat anti-human IGFBP-3 antibody was purchased from Diagnostic Systems Laboratories (Webster, TX); rabbit anti-DYKDDDDK (FLAG tag) and rabbit anti-caspase-3 antibodies were from Cell Signaling Technology (Danvers, MA). The mouse anti-β-actin and mouse anti-Hsp60 antibodies, pCMV-FLAG expression vector, and the CelLytic NuCLEAR cell fractionation kit were purchased from Sigma (St. Louis, MO). Mouse anti-DNA-PK catalytic subunit antibody was from Kamiya (Seattle, WA). I-Block was purchased from Applied Biosystems

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(Foster City, CA). The rabbit anti-RXRα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TBP (nuclear loading control) antibody was purchased from Abcam (Cambridge, MA). Pfx DNA polymerase, T4 DNA ligase, LipofectAMINE 2000 transfection reagent, and all cell culture reagents were purchased from Invitrogen (Carlsbad, CA). NU7026, horseradish peroxidase–conjugated rabbit anti-goat, goat anti-rabbit, and goat anti-mouse secondary antibodies were from Calbiochem (San Diego, CA). The rabbit anti-phosphorylated serine/threonine antibody was from Chemicon (Temecula, CA). SDS-PAGE precast gels and blotting equipment were purchased from Bio-Rad (Hercules, CA). Restriction enzymes were from Fermentas (Hanover, MD). Recombinant DNA-PK and its substrate, ATP, CellTiter 96 AQueous One Solution Cell Proliferation Assay, and Apo-ONE Homogeneous Caspase-3/7 Assay were purchased from Promega (Madison, WI).

Cloning and mutagenesis. The putative DNA-PK phosphorylation sites of IGFBP-3 (Ser^{156}, Ser^{165}, and Thr^{170}) have been previously described (17) and were confirmed using NetPhos program in the CBS prediction servers (20). The three putative phosphorylation sites were individually mutated to alanine to prevent their phosphorylation. IGFBP-3 in PBS was mutated using PCR-based mutagenesis (sense primers: S156A, 5′-AAGAAAGGGCATGCTAAAGACGCCCAGCGCTACAAAGTTGACTACGAGTCTCA-3′; S165A, 5′-AGCCAGCGCTACAAAGTTGACTACGAGGCTCAGAGCACAGATACCCAGAACTT-3′; T170A, 5′-AAAGTTGACTACGAGTCTCAGAGCACAGATGCCCAGAACTTCTCCTCCGAGTCCAA-3′ and their reverse complement antisense copies) using Pfx DNA polymerase. Template DNA was digested using *Dpn*I (Fermentas), and all constructs (termed BP3, 156A, 165A, and 170A) were cloned into pCMV-FLAG (Sigma).

***In vitro* phosphorylation assay.** IGFBP-3 was phosphorylated *in vitro* by DNA-PK in the presence of ATP following the manufacturer's instructions. Phosphorylated IGFBP-3 was then analyzed by SDS-PAGE followed by phospho-specific immunoblotting.

Cell culture. The LAPC4 prostate cancer cell line was a generous gift from Charles Sawyers (University of California at Los Angeles, Los Angeles, CA). LAPC4 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 nmol/L R1881 (Perkin-Elmer Life Sciences, Wellesley, MA). 22RV1 prostate carcinoma cell line [American Type Culture Collection (ATCC), Manassas, VA] was maintained in RPMI 1840 supplemented with 10% FBS and 1% penicillin/streptomycin. M059K and M059J glioblastoma cell lines (ATCC) were cultured in F-12/DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids. For individual experiments, cells were seeded at a final density of $1 \times 10^5/cm^2$ in 96-well, six-well, or 10-cm plates and grown to 80% confluence in a humidified atmosphere of 5% CO_2 at 37°C before treatment. All treatments were carried out as indicated in serum-free medium.

Transient transfection. Cells growing on six-well plates were transfected using LipofectAMINE 2000 following the manufacturer's instructions. Briefly, 4 μg DNA was diluted in serum-free medium and combined with LipofectAMINE transfection reagent. Complexes were applied to cells in culture and incubated for 24 to 48 hours before analysis.

Immunoblotting. Cell lysates containing 20 μg protein were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in 0.2% I-Block in PBS containing 0.1% Tween 20 for 3 hours at room temperature and then probed with the appropriate primary and secondary antibodies. Antibody-antigen complexes were visualized by Western Lightning Chemiluminescence reagents (Perkin-Elmer Life Sciences) and autoradiography.

Cell fractionation. Cells on 10-cm plastic dishes were treated as indicated. Nuclear and cytoplasmic fractions were harvested using CelLytic NuCLEAR cell fractionation kit following the manufacturer's instructions. Separated fractions were quantified and analyzed by SDS-PAGE. Validity of separation was determined by immunoblotting for TBP and Hsp60.

Immunoprecipitation. Cell lysate (50 μg) or conditioned medium (10 mL) was incubated with 5 μL goat anti-human IGFBP-3 antibody overnight at 4°C. Protein A-Sepharose (50 μL, 25%) was added and samples were incubated at 4°C for 1 hour. Bound protein was eluted in Laemmli sample buffer [60 mmol/L Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromphenol blue], and the phosphorylation status of IGFBP-3 was assessed by immunoblotting with phosphorylated-specific antibodies. For coimmunoprecipitation experiments, samples were immunoprecipitated as above and analyzed by SDS-PAGE followed by immunoblotting.

Analysis of apoptosis. Apoptosis was assessed in cells growing in 96-well plates using Apo-ONE Homogeneous Caspase-3/7 Assay following the manufacturer's instructions.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell proliferation assay. To assess cell viability/proliferation, cells growing in 96-well plates were treated as appropriate and analyzed by CellTiter 96 AQueous One Solution Cell Proliferation Assay following the manufacturer's instructions.

Statistical analysis. Statistical analyses were analyzed using Student's t test and are presented as mean ± SE. Differences were considered statistically significant when $P < 0.05$.

Results

IGFBP-3 is phosphorylated by DNA-PK *in vitro*. It has been reported that adenoviral-derived IGFBP-3 can be phosphorylated by DNA-PK in the presence of [γ-^{32}P]ATP or using HeLa cell extract in the presence of a DNA-PK-specific substrate (17). To confirm our ability to detect *in vitro* DNA-PK-phosphorylated IGFBP-3, we incubated recombinant DNA-PK with recombinant IGFBP-3 in the presence of ATP. The resulting proteins were separated by SDS-PAGE and analyzed by phosphorylated-specific serine/threonine and IGFBP-3 antibodies. Phosphorylated-specific (serine/threonine) antibodies recognize IGFBP-3 only after incubation with DNA-PK, confirming that DNA-PK phosphorylates IGFBP-3 and that generic phosphorylated antibodies can be used to detect phosphorylated IGFBP-3 (Fig. 1*A*). We set out to confirm the phosphorylation of IGFBP-3 by DNA-PK *in vitro* using two systems. First, 22RV1 prostate cancer cells were incubated with 2 μg/mL IGFBP-3 in the presence and absence of 10 μmol/L NU7026, a specific ATP-competitive inhibitor for DNA-PK cells. IGFBP-3 was immunoprecipitated from cell lysates, and its phosphorylation status was analyzed by phosphorylated-specific immunoblotting. In the presence of NU7026, serine/threonine phosphorylation of both exogenously added (nonglycosylated, 29 kDa) and endogenous IGFBP-3 (glycosylated, 44 kDa) was reduced >3-fold (Fig. 1*B*). To confirm these observations, we used a paired cell system of glioblastoma cell lines that either lack (M059J) or express (M059K) DNA-PK (21). Endogenous IGFBP-3 was immunoprecipitated from M059K and M059J cells after 24 hours of incubation in serum-free medium in the presence or absence of NU7026, and phosphorylation was assessed using phosphorylated serine/threonine immunoblotting. Three-fold reduced phosphorylation of IGFBP-3 was observed in M059J cell lysates compared with M059K (Fig. 1*C*). In addition, phosphorylation of IGFBP-3 in M059K, but not M059J, cells was partially inhibited by coincubation with NU7026, confirming that IGFBP-3 is phosphorylated by DNA-PK *in vitro*. To determine whether secreted IGFBP-3 has been phosphorylated by DNA-PK, we incubated 22RV1 cells in serum-free medium in the presence or absence of NU7026 for 24 hours. IGFBP-3 was immunoprecipitated from conditioned medium, and its phosphorylation status was assessed by phosphorylated-specific immunoblotting. Similar amounts of both total and phosphorylated IGFBP-3 were detected in conditioned medium regardless of the presence of NU7026, suggesting that DNA-PK phosphorylation of IGFBP-3 does not occur during its secretion and that secreted IGFBP-3 does not get phosphorylated by DNA-PK in 22RV1 prostate cancer cells (Fig. 1*D*).

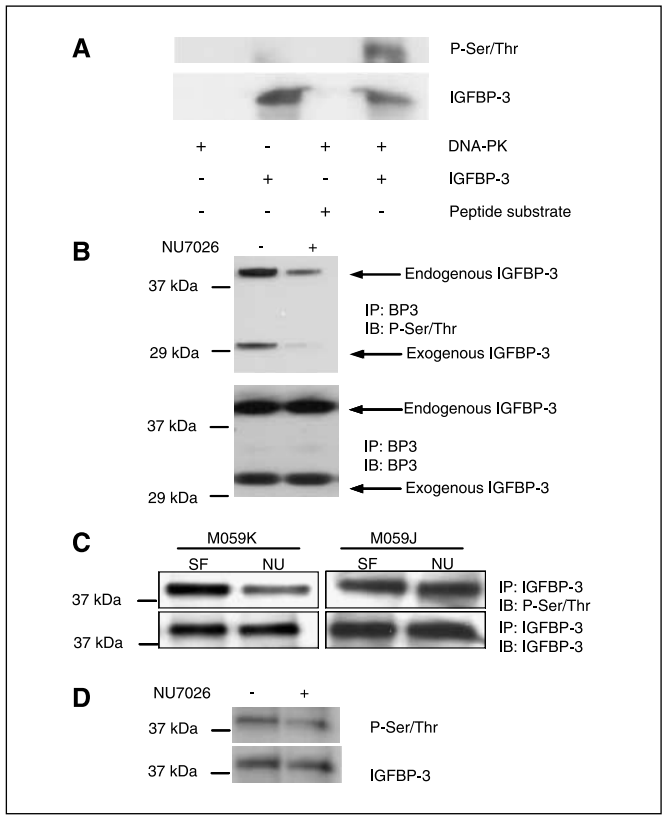

Figure 1. Reduced phosphorylation of IGFBP-3 in the absence of DNA-PK activity. *A,* recombinant DNA-PK was incubated with or without its peptide substrate or IGFBP-3 and ATP. Protein mixtures were separated by SDS-PAGE, and the phosphorylation status of IGFBP-3 was assessed by immunoblotting with phosphorylated serine/threonine (*P-Ser/Thr*) antibodies. *B,* 22RV1 cells were incubated in serum-free medium for 24 hours followed by 24 hours of treatment with 2 μg/mL IGFBP-3 and/or 10 μmol/L NU7026. The phosphorylation status of IGFBP-3 was assessed by immunoprecipitation (*IP*) with anti-IGFBP-3 followed by reducing SDS-PAGE and immunoblotting (*IB*) for phosphorylated serine/threonine (*top*) and IGFBP-3 (*bottom*). *C,* M059K and M059J glioblastoma cells were incubated in serum-free (*SF*) medium for 24 hours. Immunoblot for phosphorylated serine/threonine (*top*) and IGFBP-3 (*bottom*) after immunoprecipitation for IGFBP-3 followed by reducing SDS-PAGE. Each blot is representative of three independent experiments. *D,* phosphorylation status of IGFBP-3 secreted from 22RV1 cells incubated in serum-free medium in the presence or absence of NU7026 for 24 hours was assessed in conditioned medium as in (*B*).

Phosphorylation of IGFBP-3 by DNA-PK is necessary for its growth-inhibitory and apoptosis-inducing actions. IGFBP-3 directly inhibits proliferation (22, 23) and induces cell death in many tumor cell types, including prostate, lung, colon, and breast cancers (2, 3, 24, 25). Many post-translational modifications of IGFBP-3 have been reported, including phosphorylation by kinases, such as DNA-PK (15, 17). However, little physiologic relevance for such modifications is understood. Potential effects of DNA-PK activity on the ability of IGFBP-3 to inhibit cell growth were investigated by incubating LAPC4 cells with increasing concentrations of IGFBP-3 (0, 1, 2, and 4 μg/mL) in the presence or absence of NU7026 for 72 hours in serum-free medium. Treatment of LAPC4 cells with IGFBP-3 significantly inhibited cell growth in a dose-dependent manner (Fig. 2). However, coincubation with NU7026 completely prevented the growth-inhibitory actions of IGFBP-3. This suggests that phosphorylation of IGFBP-3 by DNA-PK is essential for its antiproliferative actions in prostate cancer. To determine whether phosphorylation of IGFBP-3 by DNA-PK plays a role in enhancing or inhibiting its apoptotic actions, we incubated 22RV1 and LAPC4 prostate cancer cells with 2 μg/mL human recombinant nonglycosylated IGFBP-3 ± 10 μmol/L NU7026 and assessed apoptosis induction using a fluorogenic caspase-3/caspase-7 substrate. In both cell types, treatment with exogenous IGFBP-3 caused a 40% increase in cleavage of caspase substrate compared with serum-free control ($P < 0.05$ in LAPC4; $P < 0.01$ in 22RV1; Fig. 3*A*). However, when cells were incubated with IGFBP-3 in the presence of the DNA-PK inhibitor NU7026, apoptosis induction by IGFBP-3 was completely abrogated and levels of caspase substrate cleavage were comparable with control cells. Incubation of either LAPC4 or 22RV1 cells with NU7026 alone caused no increase in caspase activity compared with serum-free controls, suggesting that NU7026 inhibits apoptosis induction by IGFBP-3 directly by inhibiting its phosphorylation as opposed to an indirect cellular effect.

To confirm these findings, we assessed caspase-3/caspase-7 activity in M059K and M059J cells incubated in the presence and absence of 2 μg/mL IGFBP-3 for 24 hours. The addition of IGFBP-3 to M059K cells led to a 30% increase in caspase activation ($P < 0.05$; Fig. 3*B*). In contrast, M059J cells, which completely lack the catalytic subunit of DNA-PK, have no significant response to treatment with IGFBP-3 (Fig. 3*B*). Taken together, these data suggest that DNA-PK activity is essential for the growth-inhibitory and apoptosis-inducing actions of IGFBP-3.

Phosphorylation of IGFBP-3 by DNA-PK enhances nuclear accumulation and is essential for interactions with RXRα. We have previously shown that the apoptosis-inducing actions of IGFBP-3 require its internalization, nuclear localization, and interaction with the nuclear receptor RXRα (10, 11). Because phosphorylation of IGFBP-3 by DNA-PK is also essential for its apoptosis-inducing actions, we hypothesized that phosphorylation of IGFBP-3 is necessary for its interaction with RXRα. 22RV1 prostate cancer cells were incubated for 24 hours in serum-free medium in the presence or absence of NU7026, and cytoplasmic and nuclear fractions were isolated. IGFBP-3 immunoblotting showed impaired nuclear localization of IGFBP-3 after treatment with NU7026 (Fig. 4*A*). To determine whether impaired nuclear

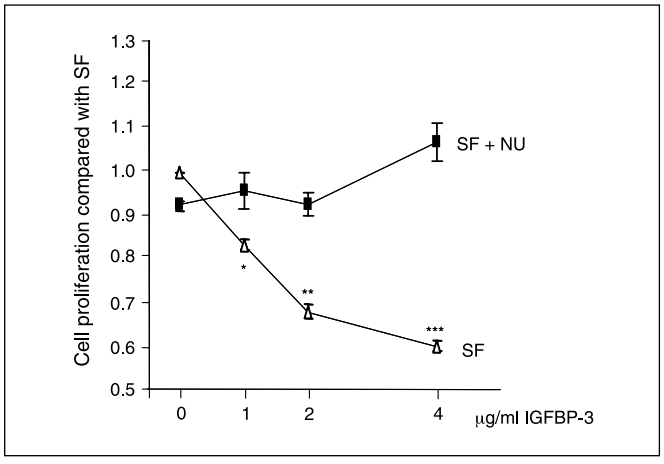

Figure 2. Growth inhibition by IGFBP-3 requires phosphorylation by DNA-PK. LAPC4 cells were incubated with increasing concentrations of exogenous IGFBP-3 for 72 hours in serum-free medium in the presence and absence of 10 μmol/L NU7026 (*NU*). Cell proliferation was assessed by enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a formazan product. $n = 4$. Significance that mean is different from 1 (untreated control): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

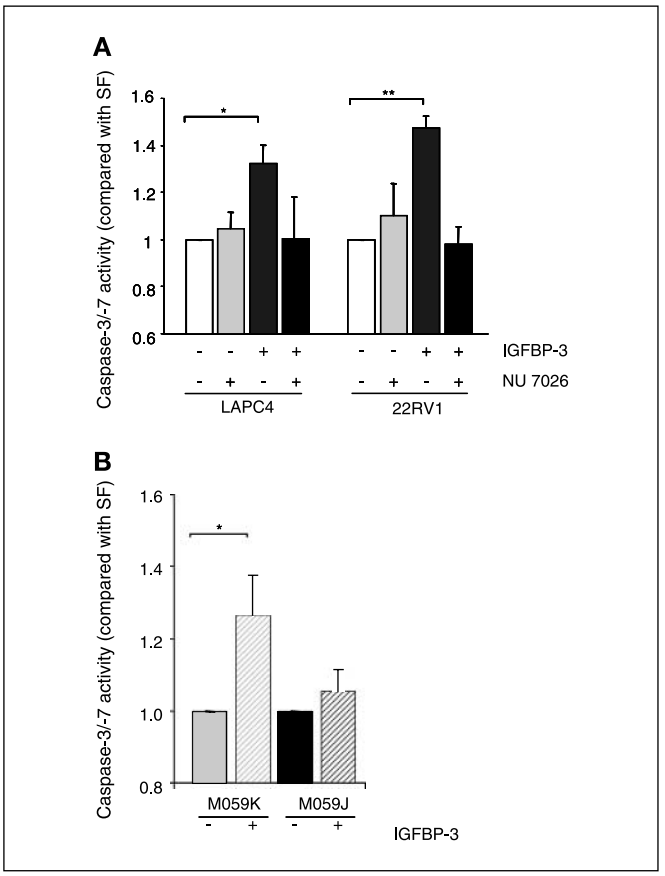

Figure 3. Apoptosis induction by IGFBP-3 requires phosphorylation by DNA-PK. *A,* LAPC4 and 22RV1 cells were incubated in serum-free medium for 24 hours followed by treatment with 2 μg/mL IGFBP-3 for 24 hours in the presence and absence of 10 μmol/L NU7026. Apoptosis was assessed by cleavage of a fluorogenic caspase-3/caspase-7 substrate. *B,* M059K and M059J cells were incubated in serum-free medium for 24 hours followed by treatment with 2 μg/mL IGFBP-3 for 24 hours. Apoptosis was measured as in (*A*). $n = 3$. Significance that mean is different from 1: *, $P < 0.05$; **, $P < 0.01$.

localization resulted in reduced RXRα binding, we incubated 22RV1 cells with 2 μg/mL IGFBP-3 in the presence and absence of 10 μmol/L NU7026 for 24 hours in serum-free medium and harvested whole-cell extracts. IGFBP-3 was immunoprecipitated from lysates and analyzed by SDS-PAGE. Coimmunoprecipitation of RXRα was detected by immunoblotting in cells incubated with IGFBP-3 alone (Fig. 4*B*). However, RXRα was no longer detected in the IGFBP-3 immunoprecipitation complex when cells were coincubated with IGFBP-3 and NU7026, correlated with reduced serine/threonine phosphorylation, suggesting that inhibiting the phosphorylation of IGFBP-3 by DNA-PK prevents its interaction with RXRα. These data were confirmed by assessing the ability of IGFBP-3 to interact with RXRα in M059K and M059J cell lines (Fig. 4*C*). When incubated with exogenous IGFBP-3, RXRα could be coimmunoprecipitated from M059K but not M059J cells, correlated with the phosphorylation status of IGFBP-3. When M059K cells were preincubated with NU7026, IGFBP-3 and RXRα no longer coimmunoprecipitated, confirming that DNA-PK activity is necessary for this interaction to occur. This provides a potential mechanism for the lack of apoptosis induction by IGFBP-3 observed in the absence of active DNA-PK.

Phosphorylation of Ser^{156} is critical for apoptosis induction by IGFBP-3. A cluster of three potential DNA-PK phosphorylation sites (Q/E/D-S/T-Q) have been identified in the central nonconserved domain of IGFBP-3, Ser^{156}, Ser^{165}, and Thr^{170}, which are highly conserved among human, mouse, rat, bovine, and porcine IGFBP-3 (17, 26). To determine the contribution of each residue to the functional regulation of the apoptotic actions of IGFBP-3, we mutated each residue individually to alanine by PCR-based site-directed mutagenesis. We then assessed the ability of pCMV-IGFBP-3-FLAG, pCMV-IGFBP-3/S156A-FLAG, pCMV-IGFBP-3/S165A-FLAG, and pCMV-IGFBP-3/T170A-FLAG expression to induce apoptosis in LAPC4 and 22RV1 prostate cancer cells. The expression of transfected constructs was verified by IGFBP-3 immunoblotting (Fig. 5*A*). Equivalent levels of endogenous IGFBP-3 (lower band) were detected in control cell lysates and in cells transfected with IGFBP-3. Slightly higher molecular weight IGFBP-3 was detected in cell lysates transfected with all forms of IGFBP-3 but was absent in pCMV-FLAG control transfected cells, corresponding to FLAG-tagged transfected constructs. Equivalent expression of all forms of transfected IGFBP-3 was observed. We assessed the ability of IGFBP-3/S156A, IGFBP-3/S165A, and IGFBP-3/T170A to induce apoptosis compared with wild-type IGFBP-3 by evaluating caspase-3/caspase-7 activity in transfected LAPC4 cells. Transfection of wild-type IGFBP-3 caused a 60% increase in apoptosis compared with control transfected cells ($P < 0.01$) that was completely abrogated by incubation with 10 μmol/L NU7026 (Fig. 5*B*). Similarly, LAPC4 cells transfected with either

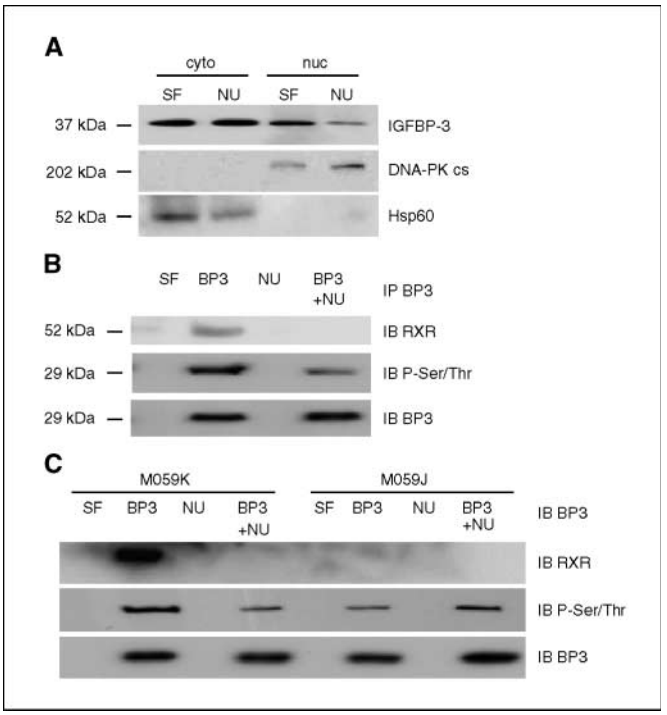

Figure 4. Preventing phosphorylation by DNA-PK reduces nuclear localization of IGFBP-3 and prevents interaction with RXRα. *A,* 22RV1 cells were incubated with and without 10 μmol/L NU7026 for 24 hours in serum-free medium. The intracellular localization of endogenous IGFBP-3 was assessed by anti-IGFBP-3 immunoblot after fractionation of nuclear (*nuc*) and cytoplasmic (*cyto*) fractions and SDS-PAGE. Validity of fractionation was confirmed by immunoblotting for Hsp60 (cytoplasmic fraction) and DNA-PKcs (nuclear fraction). The ability of IGFBP-3 to bind to RXRα in the absence of DNA-PK activity was assessed by immunoblotting for RXRα after immunoprecipitation with anti-IGFBP-3 in 22RV1 (*B*) or M059K/M059J (*C*) cells incubated in serum-free medium for 24 hours followed by treatment with 2 μg/mL IGFBP-3 for 24 hours in the presence and absence of 10 μmol/L NU7026. Blots are representative of three independent experiments.

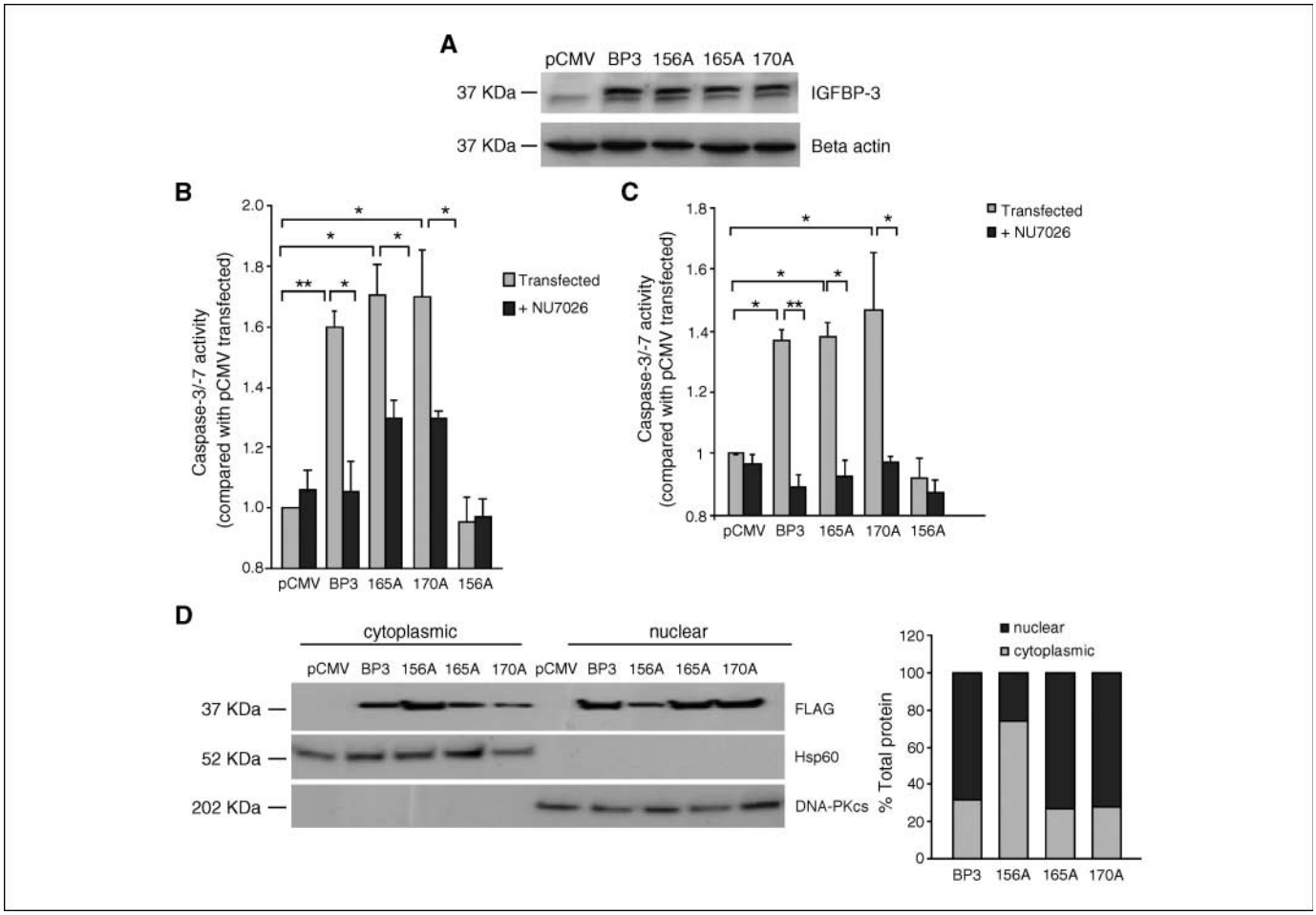

Figure 5. Phosphorylation of Ser^{156} is critical for apoptosis induction by IGFBP-3. Prostate cancer cells were transiently transfected with pCMV-FLAG (*pCMV*), pCMV-IGFBP-3-FLAG (*BP3*), pCMV-IGFBP-3/S156A-FLAG (*156A*), pCMV-IGFBP-3/S165A-FLAG (*165A*), or pCMV-IGFBP-3/T170A-FLAG (*170A*). *A,* immunoblot for IGFBP-3 (*top*) and β-actin (*bottom*, loading control) in 22RV1 cells transfected as above and harvested after 24 hours. Caspase-3/caspase-7 activity in LAPC4 (*B*) and 22RV1 (*C*) cells transfected as above and assayed after 24 hours. $n = 3$. Significance that mean is different from 1: *, $P < 0.05$; **, $P < 0.01$. *D,* 22RV1 cells were transfected as above and nuclear and cytoplasmic fractions were harvested after 24 hours. Immunoblot for FLAG (*top*), Hsp60 (*middle*, cytoplasmic control), and TBP (*bottom*, nuclear control). *Right,* quantification of FLAG immunoblot to show proportion of nuclear versus cytoplasmic protein.

pCMV-IGFBP-3/S165A or pCMV-IGFBP-3/T170A caused a 60% increase in caspase-3/caspase-7 activity that was also inhibited by coincubation with NU7026 ($P < 0.05$). In contrast, pCMV-IGFBP-3/S156A was unable to promote caspase activation either in the presence or in the absence of NU7026. To confirm these observations, we also analyzed caspase-3/caspase-7 activity in transfected 22RV1 cells (Fig. 5*C*). Cells transfected with wild-type IGFBP-3, IGFBP-3/S165A, or IGFBP-3/T170A displayed ~40% increased levels of caspase-3/caspase-7 activity compared with control transfected cells ($P < 0.01$ and $P < 0.05$, respectively). The increased caspase activation was inhibited by coincubation with NU7026. In contrast, 22RV1 cells overexpressing IGFBP-3/S156A displayed comparable levels of caspase activation with control cells and were unaffected by the addition of NU7026. Together, these studies suggest that phosphorylation of Ser^{156} by DNA-PK is essential for the apoptosis-inducing actions of IGFBP-3 in prostate cancer cells. Incubation of 22RV1 cells with NU7026 causes reduced nuclear localization of IGFBP-3 (Fig. 4*A*). To determine if reduced nuclear expression of IGFBP-3/S156A occurred, we transiently transfected 22RV1 cells with pCMV-FLAG, pCMV-IGFBP-3, pCMV-IGFBP-3/S156A, pCMV-IGFBP-3/S165A, and pCMV-IGFBP-3/T170A and isolated nuclear and cytoplasmic fractions. Cellular localization of transfected IGFBP-3 was assessed by SDS-PAGE followed by FLAG immunoblotting (Fig. 5*D*). Consistent with data obtained using NU7026 (Fig. 4*A*), we observed 3-fold reduced nuclear accumulation of IGFBP-3/S156A compared with wild-type IGFBP-3, IGFBP-3/S165A, and IGFBP-3/T170A. Validity of fractionation was confirmed by immunoblotting for Hsp60 (cytoplasmic fraction) and TBP (nuclear fraction). As expected, no FLAG immunoreactivity was detected in control transfected cell lysates. These data identify phosphorylation of Ser^{156} by DNA-PK as critical for the apoptotic actions of IGFBP-3 in prostate cancer cell lines.

Discussion

In addition to its role as the principal serum carrier of IGFs, IGFBP-3 also functions to potentiate and inhibit IGF action by regulating the bioavailability of IGFs to interact with the IGF type I receptor (1). In this way, IGFBP-3 can both ameliorate and abrogate IGF-stimulated cell proliferation and survival. Beyond its role of modulating IGF action, IGF-independent actions of IGFBP-3 have been described. For example IGFBP-3 is known to promote apoptosis in an IGF-independent manner in many cancer models

(2, 3, 24, 25). In addition, the absence of apoptosis in senescent fibroblasts has been associated with the absence of nuclear IGFBP-3 (27). Interestingly, human papillomavirus type 16 E7 oncoprotein, which can override senescence to immortalize human primary cells, can directly bind to and target IGFBP-3 for degradation (28). Several other cellular binding partners for IGFBP-3 have also been identified, including RXRα and humanin (11, 29, 30). Although factors, including p53, vitamin D, and transforming growth factor-β, are known to regulate IGFBP-3 expression (19, 31, 32), the mechanism of action for many IGF-independent roles of IGFBP-3 and how such functions are regulated are poorly understood.

Protein phosphorylation and dephosphorylation are common mechanisms for regulating the activity of numerous proteins and transcription factors in response to changing stimuli and environmental conditions (33). Of the six IGFBPs, phosphorylation has been reported for IGFBP-1 and IGFBP-3 (14–17, 34, 35). Previous reports of the effects of phosphorylation on IGFBP-3 action have suggested that post-translational modification of IGFBP-3 in this way may play a role in the regulation of IGF binding and nuclear localization (16, 17). Interestingly, phosphorylation has been reported to both enhance (16) and inhibit (17) IGF binding by IGFBP-3. Although these seem to be conflicting reports, it is possible that these are cell-specific effects or that different kinases play distinct roles in enhancing or preventing IGF-IGFBP binding. We have now identified phosphorylation of Ser^{156} of IGFBP-3 to be a critical step in the induction of apoptosis by IGFBP-3 in prostate cancer cells.

We have described the relevance of phosphorylation by DNA-PK for the roles of IGFBP-3 in prostate cancer. However, it is unclear what role phosphorylation by other kinases may play in regulating IGFBP-3 action. *In vitro* phosphorylation has been described by PKA and at residues consistent with consensus CK2 phosphorylation sites (14, 15, 17). Coverley et al. (15) showed an 80% decrease in [^{32}P]phosphate incorporation in CHO cells transfected with IGFBP-3 in which Ser^{111} and Ser^{113} (potential CK2 phosphoacceptor sites) had been mutated to alanine. In addition to suggesting that Ser^{111} and Ser^{113} can be phosphorylated, these data also suggested that other residues in IGFBP-3 are also phosphorylated. Similarly, comparing the phosphorylation status of IGFBP-3 in the presence and absence of active DNA-PK reveals partial but not complete reduction of phosphorylation without active DNA-PK, again suggesting that phosphorylation of IGFBP-3 by multiple kinases may occur. Although Ser^{111}/Ser^{113} phosphorylation may influence IGF binding by IGFBP-3, what significance phosphorylation by CK2, PKA, DNA-PK, and other unidentified kinases may have on other actions of IGFBP-3 is yet to be determined.

IGFBP-3 interacts with its nuclear partner RXRα to induce apoptosis in prostate cancer cells (11) in a nuclear localization–dependent manner. However, recent reports have revealed that IGFBP-3 is also able to induce apoptosis independent of nuclear localization. For example, a form of IGFBP-3 with a mutated NLS, which was unable to interact with the cell membrane and had impaired internalization, was still able to promote apoptosis in breast cancer cells (13). This suggests that IGFBP-3 is also able to promote apoptosis without being internalized [e.g., by interacting with a specific extracellular receptor (36)]. It is therefore possible that IGFBP-3 can function in different ways to promote cell death in cancer cells possibly in a cell type–specific manner.

Because DNA-PK is also predominantly a nuclear protein, it is likely that the phosphorylation of IGFBP-3 by DNA-PK occurs in the nucleus, promoting the association of IGFBP-3 with RXR and resulting in the induction of apoptosis. As Ser^{156} is in a region of IGFBP-3 distinct from the RXR-binding domain, it is possible that phosphorylation causes a conformational change in IGFBP-3 to facilitate interaction with RXRα. Such a mechanism would support data describing that nuclear localization is necessary for apoptosis induction by IGFBP-3 (11, 12). However, although DNA-PK acts predominantly as a nuclear kinase, low levels have been reported in cytoplasmic extracts derived from HTC rat hepatoma and HeLa cells (17, 37, 38), suggesting that phosphorylation of IGFBP-3 by DNA-PK may indeed occur in the cytoplasm or at the cell membrane. Indeed, phosphorylation of Akt by DNA-PK has been reported to occur at the cell membrane (39), suggesting that IGFBP-3 could potentially be phosphorylated during secretion or cellular uptake. However, our data suggest it is unlikely that phosphorylation by DNA-PK occurs during secretion in prostate cancer cells because there was little difference in phosphorylation status detected in IGFBP-3 from the conditioned medium of incubated with or without NU7026. However, phosphorylation by other kinases may occur either at the cell membrane or during secretion because phosphorylated IGFBP-3 is detectable in conditioned medium.

DNA-PK belongs to a family of large phosphatidylinositol 3-kinase-like proteins, which also includes ataxia-telangiectasia mutated and FRAP (40). Intracellular targets of DNA-PK kinase activity include p53, Mdm2, RNA polymerase II large subunit, and chromatin components (18, 41, 42). Functional DNA-PK consists of a catalytic subunit (DNA-PKcs) and a DNA-targeting heterodimer, Ku (43). Ku is tightly associated with DNA and functions by stimulating DNA-PKcs kinase activity toward DNA-bound targets and functions most effectively when the target protein is bound to the same DNA strand as DNA-PK itself (43, 44). Because DNA-PK requires DNA for its kinase activity, the demonstration of phosphorylation of IGFBP-3 by DNA-PK by ourselves and others would therefore seem to support growing evidence for either a direct or indirect role of IGFBP-3 in DNA binding and the modulation of gene transcription.

In summary, phosphorylation of IGFBP-3 by DNA-PK at Ser^{156} is a critical step in the cellular functions of IGFBP-3 in modulating apoptosis and growth inhibition. The generation of a novel nonphosphorylated mutant of IGFBP-3 will provide a crucial tool for future study of the biological actions of IGFBP-3.

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